BIOLOGICAL

Susceptibility of the Cottonwood Leaf Beetle (Coleoptera: Chrysomelidae) to Different Strains and Transgenic Toxins of *Bacillus thuringiensis*

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ABSTRACT *Populus* spp. (which include cottonwoods, aspens, and poplars) are important sources of wood, wood fiber (pulp), and biofuels throughout the world, and are often intensively managed in short rotation stands. The cottonwood leaf beetle, *Chrysomela scripts* F., is a major pest of Populus throughout North America. It would be difficult to breed insect resistance into these trees using traditional plant breeding techniques because of their long generation time (4-8 yr); however, insect resistance could be produced through genetic engineering. Toxins from *Bacillus thuringiensis* (Berliner) genes have negligible nontarget effects and are amenable to genetic engineering. We tested the toxicity of 16 *B. thuringiensis* preparations to identify genes that produce toxins effective against the cottonwood leaf beetle. B. *thuringiensis* preparations that contained spores were found to be only moderately more virulent than isolated toxins. Strains that produced Cry3A, Cry3B, and Cry8B caused 97-100% mortality in 1st and 2nd instars, with a mean time to death of 1-5 d. Mature larvae were less susceptible to these toxins than were neonates; however, the toxicity of 1 moderately active strain was not affected by larval age. Adults were not as sensitive as larvae to any of these toxins. Cropping with trees genetically engineered to produce *B. thuringiensis* toxins could lead to the evolution of toxin resistance in cottonwood leaf beetles. We found beetles from 3 different U.S. states varied significantly in their susceptibility to Cry3A. This variation means that regional differences in control levels could occur in the field, and that the potential for evolution of B. *thurwngiensfs*resistance may already exist in some populations of this insect. Therefore, it is very important that before genetically engineered poplars are used extensively, management strategies be developed and implemented to prevent the evolution of resistance to B. *thuringiensis* in the cottonwood leaf beetle.

WORLD DEMANDS FOR wood fiber, fuel, and timber have increased greatly, placing great pressure on the harvest of natural forests. In response to this demand, many pulp and paper companies are producing their own wood pulp using plantations of fast-growing hardwoods such as Populus spp. (including cottonwoods, aspens, and poplars). These trees are intensively cultivated and grown in rotation cycles consisting of S-7 yr (Dickman and Stuart 1983), often in former crop and pasture lands. Populus trees grown in plantations also serve as a source of structural wood and are used as an alternative to coal and oil for energy production (Hohenstein and Wright 1994, Hall 1997).

The cottonwood leaf beetle, *Chrysomela scripts F.,* is a major pest of cottonwoods and poplars throughout the continental United States. It causes the greatest damage during the 1st 2-3 yr of tree growth (Bingaman and Hart 1992). This pest has 2-5 generations per year, depending on the climate, and both larvae and adults feed on tree foliage and meristematic tissue. Currently, C. *scripts is* controlled primarily with dimethoate and carbaryl, but is susceptible to the Cry3A toxin of *Bacillus thuringiensis* (Berliner) variety *tenehrionris* (Bauer 1990, Bauer and Pankratz 1992). Cry1B, another B. *thuringiensis* toxin, also shows moderate toxicity to larvae if the toxin is first partially digested with protease (Ramachandran et al. 1993, Bradley et al. 1995).

Insect-resistant tree varieties could benefit pulp and fuel production systems greatly because these crops have only moderate values and pesticide applications are expensive. Pesticides must be applied either serially or systemically through drip irrigation systems, and the latter is not available in all production systems. Furthermore, insect resistant varieties target the pest directly and thus avoid many of the problems associated with pesticide overspray.

Populus trees require 4-8 yr to reach sexual maturity; therefore, breeding for desirable traits (such as insect resistance) through traditional techniques could take decades. Genetic engineering techniques,

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however, offer promise for a more timely development of some traits. Furthermore, genetic engineering can combine insect resistance with more complex traits that have already been developed through selective breeding, such as wood quality and growth characteristics.

We screened several strains of *B. thuringiensis* for activity toward 1st-instar cottonwood leaf beetles. A subset of strains was tested in more detail to determine their effect on different developmental stages of the insect. We also tested toxins produced by genes that had been isolated from *B. thuringiensis* and transferred to nonpathogenic bacteria. This test allowed us to compare toxin preparations with and without spores. In addition, we compared the susceptibility of different populations of cottonwood leaf beetles to Cry3A and Cry3B. Genetic variability in insect susceptibility to transgenic toxins can be an important factor in determining the potential for populations to evolve resistance to toxins in transgenic plants. Furthermore, measuring susceptibility now will give a baseline by which to monitor for changes in susceptibility of the insects after transgenic trees are planted.

Materials and Methods

Rearing Methods. Cottonwood leaf beetles were from laboratory colonies maintained <1 yr. Unless otherwise indicated, the original source of beetles was near Ice Harbor, WA. Insect colonies and bioassays were maintained in a temperature-controlled room at 24-27°C with a photoperiod of 14:10 (L:D) h. Adult beetles were maintained in 4.3-liter clear plastic boxes (Pioneer Plastics, Dickson, KY) with 40-60 adults per box. Larvae were reared in the same boxes (60-100 larvae per box), or in large petri dishes (120 cm diameter). Insects were fed freshly collected leaves from hybrid poplar (a mixture of Populus *trichocarpa x P. deltoides* and P. *deltoides x P. nigra* clones). Fresh branches were collected from trees grown in the field and greenhouse. For bioassays, we used leaves from 1-yr-old greenhouse-grown trees of `OP36T (P. *deltoides x P. nigra)* and with a leaf plastochron index (LPI) of 4-8 unless otherwise indicated. The leaf plastochron index indicates a leaf s position relative to the tip of the branch (Larson and Isebrands 1971).

B. thuringiensis Materials. The B. *thuringiensis* samples tested are listed in Table 1. B, *thuringiensis* strains refer to cultures of isolates that were not genetically altered, and consisted of lyophilized preparations containing both spores and toxin. To determine the toxicity of proteins produced by particular *cry* genes, individual genes were transferred from the parent strain into an acrystalliferous strain of *B. thuringiensis,* These samples are referred to as *B. thuringiensis* clones, and they also contained both spores and toxin. To determine the effect of toxins in the absence of B. *thuringiensis* spores, *Pseudomonas fluorescens* was used as the transgene host. P. *fluorescens* clone preparations consisted of toxin crystals encapsulated in an iodinefixed bacterial cell (Feitelson et al. 1990). Mycogen (San Diego, CA) supplied all the test materials.

Table 1. Identifications of the B. thuringiensis strains and clones used

Preparation code	Toxin genes[a]	Type of sample	Strain of origin for toxin genes
A02	NI	*B. thuringiensis* strain	—
A05	NI (2 toxins, a and b)	*B. thuringiensis* strain	—
A09	NI	*B. thuringiensis* strain	—
A10	NI	*B. thuringiensis* strain	—
A11	NI	*B. thuringiensis* strain	—
HD-73	*Cry1Ac*	*B. thuringiensis* strain	—
PS50C	*Cry8A* and *cry8B*	*B. thuringiensis* strain	—
[illegible]	*Cry3B*	*B. thuringiensis* strain	—
A03	*Cry1F*	Killed *P. fluorescens*[b]	PS81I
A06	*Cry3A*	*B. thuringiensis* clone[c]	PS122D3
B13	*Toxin a*	*B. thuringiensis* clone	A05
B14	*Toxin b*	Killed *P. fluorescens*	A05
B15	*Cry3A*	Killed *P. fluorescens*	PS122D3
[illegible]	*Cry3B*	Killed *P. fluorescens*	PS43F (sequence identical to PS86B1)
B17	*Cry8A*	*B thuringiensis* clone	PS50C
B18	*Cry8B*	*B. thuringiensis* clone	PS50C

° NI, not identified.

h The *B.* thuringiensis toxin gene was cloned into P. fluorescens: therefore, no spores were present in these preparations.

`For all *B,* thuringiensis clones, the toxin gene was

Screening Bioassays. We initially tested 8 different *B. thuringiensis* strains (A02, A05, A09, A10, All, HD73, PS50C, and PS86B1; Table 1) and 2 clones (A03 and A06; Table 1) for toxicity to 1st-instar cottonwood leaf beetles. Hybrid poplar leaves were collected and dipped in an aqueous suspension of lyophilized cultures (100-ie.g toxin ml-1 water). Leaves were air dried, and then individually placed in petri dishes (9 cm diameter). For all the preparations screened, 1st instars (1 d old) were tested with 10 larvae per dish, using 4 dishes per treatment. Mortality was read every 48 h for 6 d. The following preparations were further tested to determine the effect of insect age on susceptibility: A05, PS50C, PS86B1, and A06. For this assay, 1st instars were again tested, as well as 2nd (2 d old) and 3rd (5 d old) instars, and adults (3 d postemergence). Mortality was recorded daily until all insects had either died or pupated. Adults were monitored daily for 10 d.

In all these assays, insects were given fresh, newly treated leaves every 24-48 h as needed to provide continuous exposure to the toxin. Controls were fed leaves treated with water. The bioassays were maintained in 5.6-liter plastic boxes with tight-fitting lids (Rubbermaid, Wooster, OH) and a saturated solution of NaCI. The salt solution was used to maintain a continuous humidity of 75% RH by suspending the petri dishes over the solution with a metal screen. Each dish was considered a replicate for statistical analyses involving percentage mortality.

Estimation of Toxin Titer on Treated Leaves. Water was used to estimate the amount of B. *thuringiensis* suspension left on leaves after they were dipped. Thir

ty-eight fresh leaves were collected, weighed, individually dipped in water, drained of excess water, then weighed again. This process estimated the volume of water on a dipped leaf. Each leaf was then dried for 3 d at 60°C to measure dry biomass to determine the mass of water g-' dry leaf biomass. The amount of toxin in that volume of water was determined for each concentration of toxin. This value was used to estimate the amount of toxin that transgenic poplars must produce to obtain sufficient cottonwood leaf beetle mortality.

Activity of Toxins Expressed in Transgenic Bacteria. To test the effect of toxins isolated from the parental strains of *B. thuringiensis,* we used Cry3A, CryBA, and Cry8B produced in *B. thuringiensis* clones (preparations A06, B17, and B18, respectively); and Cry3A and Cry3B produced by P. *fluorescens* clones (B15 and B16, respectively). First-instar cottonwood leaf beetles were assayed by continuously feeding them leaves dipped in a solution of 100'i,g toxin ml-' water, and recording mortality daily until pupation, as described for the other assays above. The sample size was 4 petri dishes, with 10 larvae per dish. We tested Cry8A and Cry8B separately and in combination (using 50 ~i,g of each toxin ml-1 water).

Using 1st instars and the same bioassay system, we conducted a dose-response test for Cry3A and Cry3B. Cry3A (B15) was tested at 2.5, 5.0,10.0, 20, 40, and 80.0 Ag toxin ml-1. Cry3B (B16) was tested at 10, 20, 40, and 80 ie,g toxin m-1.

Differences in Susceptibility Among Beetle Populations. In 1996, = 300 adult and larval beetles were collected from each of 3 sites. The Virginia site was a 0.8 ha planting of 2-yr-old P. *deltoides* in Suffolk County, and beetles were collected 5 May. The Georgia site was a 0.8-ha nursery near Klaxton (Evens County) containing 1- to 2-yr-old P, *deltoides,* and beetles were collected 25 April. The 3rd site was a 6,500 ha farm near Ice Harbor, Washington, of hybrid poplars up to 7-yr-old (mostly P. *trichocarpa x P. deltoides),* and these beetles were collected 6 June. These sites had never been treated with *B. thuringiensis,;* however, the Washington site had frequently been treated with dimethoate and carbaryl to control insect pests.

For the assay, 1st instars from each population were fed leaves treated with either Cry3A (B15), Cry3B (B16), or water. The bioassay was similar to that described above for 1st instars, except the toxin concentration was 10 Ag/ ml-1. This is approximately the LCD for Cry3A when tested against larvae from Washington (based on the dose-response data presented here). Insect mortality was recorded every 24-48 h for 10 d or until pupation. The assay was replicated 3 times using the 2nd, 3rd, and 4th larval generations in the laboratory. Fifty larvae were used per replicate: 5 petri dishes of 10 larvae per dish. We maintained 250-300 adults per population in each generation.

Effects of Cry3A on Adult Feeding. Adults were fed leaf discs treated with Cry3A (B15) to determine effects on feeding rates. Leaves were treated at 25, 50, and 100 Ee.g/ml-'. Leaves (LPI 3) were collected from `24-305' (a P. *trichocarpa x P. deltoides* hybrid) grown in a greenhouse, and leaf discs were punched out with a sharpened piece of metal pipe (1.77 cm diameter). Discs were placed on a wet piece of filter paper in petri dishes to prevent drying. One disc and 1 adult beetle were placed in each dish, using 10 insects per dose. Beetles were allowed to feed for 24 h before the discs were replaced with newly treated ones. The leaf area eaten per insect was measured after 24 and 48 h of exposure. A photoscanner was used to produce a digital image of each leaf disc, and the area eaten was determined using the software program Photoshop (Adobe Systems, Mountain View, CA) for image analysis. An undamaged leaf disc was used as a standard to translate pixels to square centimeters.

Statistical Analyses. A 1-tailed Dunnett (PROC GLM, SAS Institute 1990) test was used to determine if treatments had significant effects on insect mortality (as compared with controls). A Tukey student range test (PROC GLM, SAS Institute 1990) was used to compare mortality between pairs of treatments within each experiment. The Tukey test was used to determine whether mortality differed between B. *thurin*giensis preparations, between different developmental stages for each bacterial strain, and between insect populations for Cry3A. Except when treatments were compared with controls, all mortality was adjusted as described by Abbott (1925) to account for differences in control mortality between assay runs, instars, and populations.

The Kaplan-Meir product limit estimate (PROC Lifetest, SAS Institute 1990) was used to determine the survival distributions of insects from different treatments; the mean time-to-death for insects exposed to B. *thuringiensis* or cloned toxins; and the mean time-to-pupation of survivors in the population test. A Wilcoxon test (PROC Lifetest, SAS Institute 1990) was used to test the global hypotheses that virulence (time to death) or development time of survivors (time to pupation) differed between treatments. A Bonferoni test was used to compare pairs of treatments (Hardin et al. 1996). The survivorship tests are nonparametric, and the replicate size is the same as the number of insects in the treatment group.

To test whether the effect of B, *thuringiensis* toxins on adult feeding was significant, regression analysis was used with leaf area eaten as the dependent variable, and dose as the independent variable. A squareroot transformation was used on leaf area eaten so that the variance would be independent of the mean.

Results

Screening B. *thuringiensis* Strains. Of the 10 strains tested, 6 had significant effects on insect mortality ($P < 0.05$), and 5 of those caused mortality >90% (Fig. 1). Preparations A06 (Cry3A), PS86BI (Cry3B), and PS50C (Cry8 toxins) all caused 100% mortality of 1st instars. Insect mortality declined in later developmental stages (Fig. 2), and this effect was significant ($F_{AO6}=13.7, F_{PS86B1}=8.87, F_{PS50C}=7.7, df=2, P<0.01$).
In contrast, susceptibility to A05 (which has 2 unknown toxins) was not as age sensitive in larvae; how

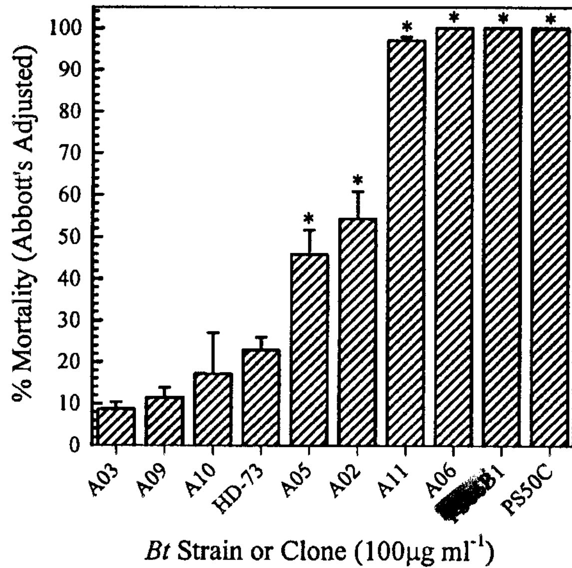

Fig. 1. Mortality of 1st-instar cottonwood leaf beetles when fed different *Bacillus thuringiensis (Bt)* preparations. Larvae were continuously fed leaves dipped in toxin-spore preparations at a concentration of 100 wg toxin ml-1 water. Lines at the top of bars represent 1 standard error of the mean (SEM) a Asterisks indicate treatments that showed significant mortality compared with the control (P < 0.05).

ever, adults were significantly (F = 3.19, df = 3, $P \leq 0.04$) less susceptible than larvae (Fig. 2).

Beetles fed A06 or PS86B1 generally died more rapidly than did those fed A05 or PS50C (Table 2).

The toxicity of Cry3A was similar whether produced by *B. thuringiensis* (A06) or *P. fluorescens* clones (B15) (Fig. 3A and 4A), except at very low doses (2 (μg/ml^{-1}), where the *B. thuringiensis* clone showed greater activity. Cry3B showed slightly diminished activity in the *P. fluorescens* clone (B16) than in the parent strain (PS86B1) (Fig. 3B and 4B). The mean time to death for Cry3B in the parent strain was always faster than in the *P. fluorescens* clone ($\chi^2_{10\ (g/ml}$ = 8.25, $\chi^2_{20\ \mu g/ml}$ = 14.58, $\chi^2_{40\ \mu g/ml}$ = 18.69, $\chi^2_{80\ \mu g/ml}$ = 37.21, df = 1, $P \leq 0.01$) (Table 3). The effect of spores enhansing on time to death was minimal for Cry3A (Table 3).

Transgenic Cry8B (B18) had activity levels indistinguishable from the spore/crystal preparation of the parent strain (PS50C) (Fig. 3C). However, Cry8A caused no significant mortality (Fig. 3C).

Treatment Levels from Leaf Dipping. We measured 1.153 (SE = 0.086) g of water per g^{-1} dry leaf biomass on dipped leaves. Treatments using 100 μg toxin ml^{-1} water would result in ≈115 μg toxin g^{-1} leaf.

Effects of *B. thuringiensis* Toxin on Adult Feeding. Cry3A (B15) significantly reduced adult feeding after 48 h (F = 12.0, $P \leq 0.001$, a = 2.62 [SE = 0.25], b = −0.58 [SE = 0.17]) (Fig. 5). Adults exposed to 100 μg ml^{-1} ate an average leaf area that was only 25% of that for control insects. The effect of Cry3A (B15) on adult feeding behavior was difficult to quantify, due to the wide variation in feeding rates that occurred even in the control treatment. The distribution of feeding rates appeared to be bimodal. At 100 jig ml-1, this bimodality disappeared, and all insects fed at a low rate.

Differences in Susceptibility Among Beetle Populations. In general, Cry3B (Bl6) was less toxic than Cry3A (B15). Cry3A was more toxic to larvae from Georgia and Virginia than to those from Washington (i.e., mortality was lower and time to death was longer for Washington beetles) (Table 4). Toxicity of Cry3B was greatest for Georgia populations, intermediate for Virginia, and lowest for Washington; however, differences were not always significant.

Control insects showed larval development rates that differed significantly among beetle populations (Table 4), with beetles from Georgia having the longest development time, followed by those from Virginia, then Washington. When insects were treated with Cry3A, survivors took significantly longer to develop than did the controls (Table 4). Interestingly, the larval development time for Cry3A survivors was similar in all 3 populations. This result means that the toxin had a greater nonlethal effect on Washington and Virginia populations than on Georgia populations. In other words, the development rate of the Georgia beetles was not delayed as much as it was for the other 2 populations.

Discussion

The cottonwood leaf beetle was highly susceptible to Cry3A, Cry3B, and CryBB. Three other *B. thurin*

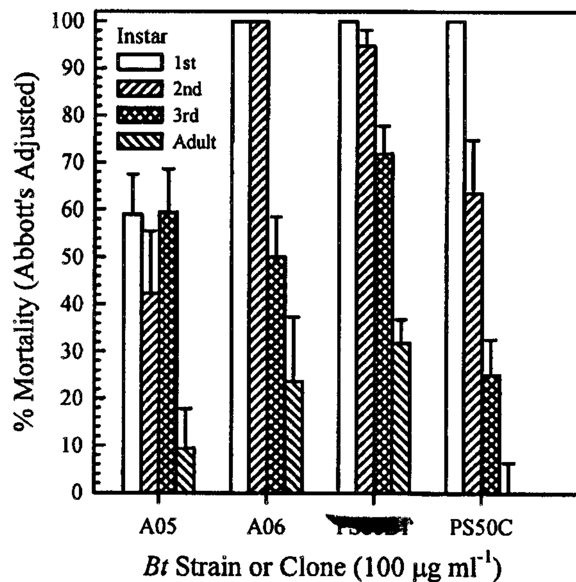

Fig. 2. The susceptibility of different developmental stages of cottonwood leaf beetle to different *Bacillus thuringiensis (Bt)* preparations. Instars indicate the developmental stage of insects at the time of 1st exposure. Insects were continuously fed leaves dipped in 100 pg toxin ml-'water for each toxin-spore preparation. Lines at the top of bars represent 1 SEM.

Table 2. Number of days (mean ± SEM) to death of cottonwood leaf beetles fed *B. thuringiensis* preparations in leaf dip assays

2nd instar exposure	*B. thuringiensis* preparation (toxins)			
	A05 (2 unknowns)	A06 (Cry3A)	PS86B1 (Cry3B)	PS50C (Cry8A and Cry8B)
1st instar	5.48 (0.48)a	1.03 (0.03)b	1.03 (0.03)b	1.85 (0.07)c
2nd instar	6.95 (0.25)b	2.25 (0.17)c	2.48 (0.26)c	3.47 (0.43)c
3rd instar	4.90 (0.19)a	4.95 (0.23)ad	4.55 (0.21)a	5.55 (0.11)d
Adult	8.33 (0.25)b	4.21 (0.29)d	3.12 (0.20)ad	8.61 (0.24)b

Leaf dip assays were done using 100 N,g toxin ml-'water. Numbers within a row or column that do not have a letter in common are significantly different (Y 5 0.05). Sample size was 40 insects per treatment.

giensis strains also had moderate to high toxicity, but the toxins from these have not yet been identified. Although *B. thuringiensis* spores increased the mortality rates caused by Cry3A and Cry3B, they were not necessary for toxicity. In our study, the strains carrying Cry3A, Cry3B, and the Cry8 toxins showed significantly higher toxicity to neonates than to mature larvae, and negligible toxicity to adults. *B. thuringiensis* often is more toxic to early developmental stages of the insect than to mature ones (e.g., McGaughey 1978, Zehnder and Gelernter 1989, Bauer 1990, Ali and Young 1996). However, early instars are not the most sensitive stage for all insects (e.g., James et a1.1993, Li et al. 1995, Liu et al. 1995) . We found cottonwood leaf beetle susceptibility to strain A05 did not vary with larval instars; therefore, the effects of age on sensitivity depend not only on the insect species, but on the B. *thuringiensis-strain as* well.

To determine if the effects we saw in the laboratory were sufficient to protect transgenic plants, we need to estimate how much toxin such plants must produce, and determine if it is reasonable to expect transgenics to produce such levels. If we assume that proteins make up ~10-17% total dry leaf biomass in woody plants (Kramer and Kozlowski 1979), our highest treatment rate was equivalent to 0.067-0.115% of the total leaf protein. Unfortunately, *B. thuringiensis* toxin production in transgenic plants varies greatly, making it difficult to predict what Populus might produce. For cotton, it ranges from 0.004 to 0.061% of extractable protein (Parrot et al. 1994), but can be as high as 0.02-2% in tomatoes (Perlak et al. 1991). Expression levels in transgenic Populus spp. have not been reported. To achieve high cottonwood leaf beetle mortality, expression levels must be at the high end of what has been seen in other plants. Furthermore, we found the products of transgenic genes to work at slower rates than toxin-spore preparations, and for some strains, total mortality was lower also. These toxins show promise for cottonwood leaf beetle control, but expression levels must be high if used in transgenic poplars.

Hassig (1995), James (1997), and James et al. (1998) discuss some of the environmental concerns associated with the wide-scale use of genetically engineered insect-resistance in Populus for fiber or fuel produc

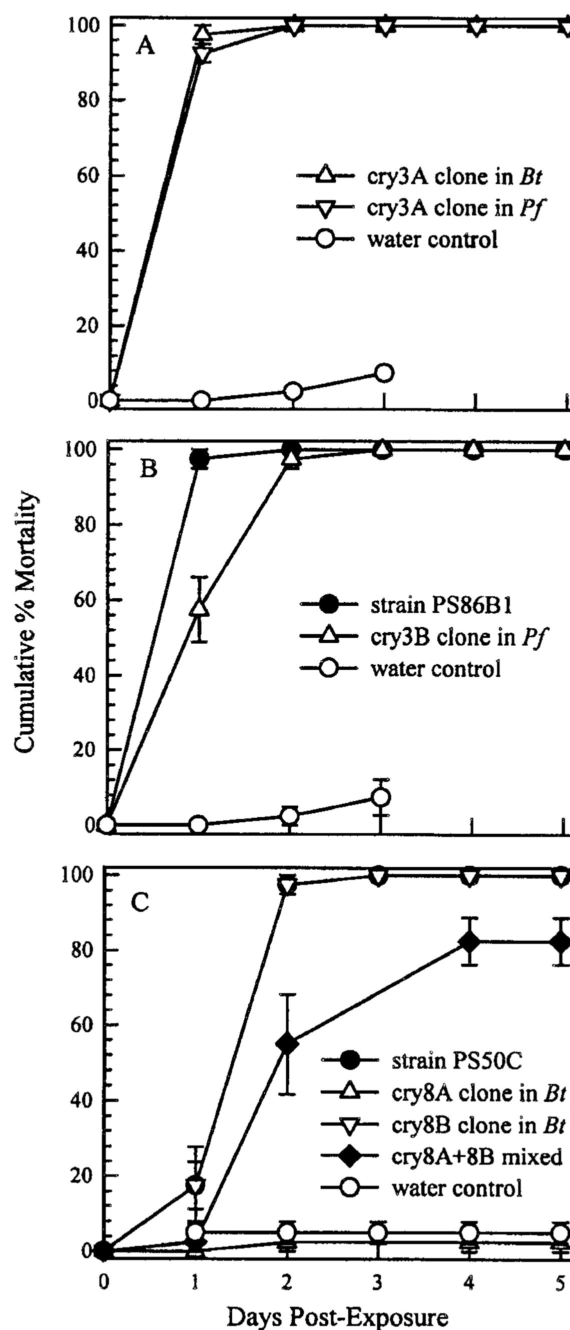

Fig. 3. Survival distribution functions of 1st-instar cottonwood leaf beetles fed either a Bacillus thuringiensis toxinspore preparation or toxin encapsulated in dead Pseudomonas fluorescens cells. All treated insects were fed leaves dipped in a preparation containing 100 p.g toxin ml-1 water. When 2 toxins were tested together, they were used at a rate of 50 N.g toxin ml-t each. Controls were fed leaves dipped in water. Bars represent SEM. (A) Cry3A toxin in a *B.* thuringiensis clone (A06) and a P. fluorescens clone (B15). (B) Strain PS86B1 and Cry3B from a P. fluorescens clone (B16). (C) Strain PS50C versus CryBA and CryBB from *B.* thuringiensis clones (B17 and B18).

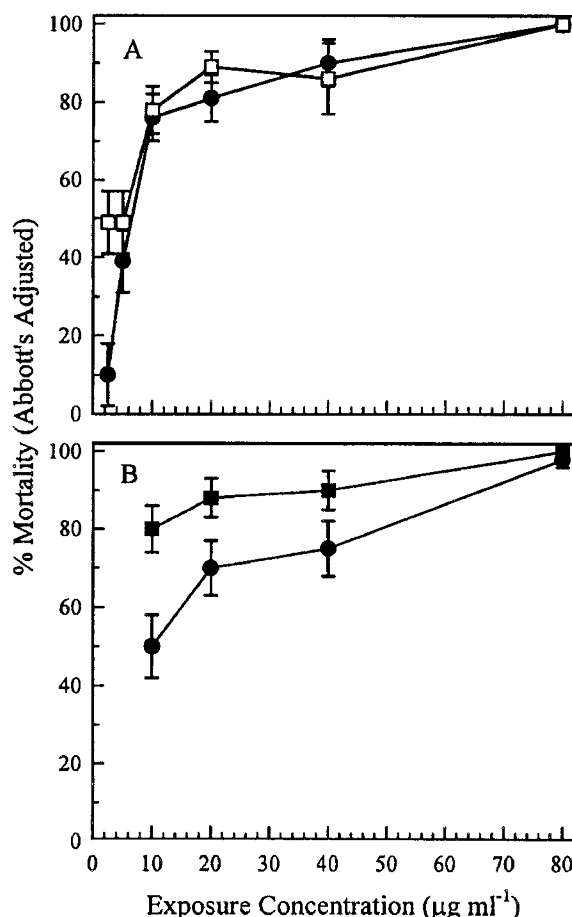

Fig.4. Dose-responses of 1st-instar cottonwood leaf beetles. (A) fed Cry3A produced by clones of • Bacillus thuringiensis and 0 *Pseudomonas fluorescens*. (B) Fed Cry3B produced by • the parent strain PS86B1 and • a Pseudomonas fluorescens clone.

tion. The environmental risks, in general, include possible negative effects on nontarget insects, the transgenic tree may be more invasive than the parent tree because of increased vigor associated with the transgenic character (i.e., insect resistance), the transgenic tree might breed with closely related wild or feral tree species and confer the transgene to the offspring. The potential for trees to become weeds may be somewhat limited, as realized through the relatively fewer weed species that are woody (James 1997). Poplars have the potential to cross-breed with wild and feral trees neighboring the plantations, however. What the ecological implications of such crosses are, and whether or not they are greater than for nontransgenic trees, is unknown. The effect of B. *thuringiensis* toxins to nontarget mammals, birds, and soil organisms that may come into contact with transgenic poplars or plant residues has been minimal in laboratory tests, as reviewed by James (1997). Furthermore, transgenic plants have the potential to replace other more toxic compounds such as clorpyrifos and dimethoate.

Table 3. Number of days to death (mean ± SEM) for 1st-instar *C. scripta* fed *B. thuringiensis* toxin-spore preparations versus transgenic toxins in leaf dip assays using 100 μg toxin ml^{-1} water. The toxin/spore preparation for Cry3A was A06, and the toxin preparation was B15. The toxin/spore preparation for Cry3B was PS86B1, and the toxin preparation was B16

Toxin	Concn, μg toxin/ml[a]	Spore preparation	Transgene toxin	Wilcoxon *P* value[b]
Cry3A	80	1.33 (0.12)	1.58 (0.11)	0.04
	40	1.90 (0.13)	2.74 (0.37)	NS
	20	1.75 (0.14)	2.65 (0.25)	0.0002
	10	3.72 (0.38)	4.65 (0.47)	NS
Cry3B	80	1.38 (0.08)	3.88 (0.48)	0.0001
	40	1.80 (0.06)	3.73 (0.28)	0.0001
	20	4.25 (0.47)	6.75 (0.45)	0.0001
	10	4.30 (0.54)	6.66 (0.61)	0.004

Sample size was 40 insects per

b P value reflects probability that the spore and toxin effects differ by chance alone. NS, not significant.

Probably the greatest concern is the potential for cottonwood leaf beetle biotypes to evolve resistance to the transgenic toxin (Raffa 1989, Bauer 1997, James 1997). The potential for resistance is fairly high considering that (1) the beetle has 3-5 generations per year; (2) the toxin will mostly likely be produced continuously by the tree, having an effect similar to a pesticide with a long residual life; and (3) this beetle has already shown a potential to develop resistance to Cry3A in laboratory studies (Bauer 1995). Thus, a carefully planned resistance management strategy is necessary if transgenic trees are to be effective over long periods. Several strategies for resistance management of transgenic plants have been proposed, and

could apply to transgenic tree crops with some modification (James 1997). Most currently accepted strat

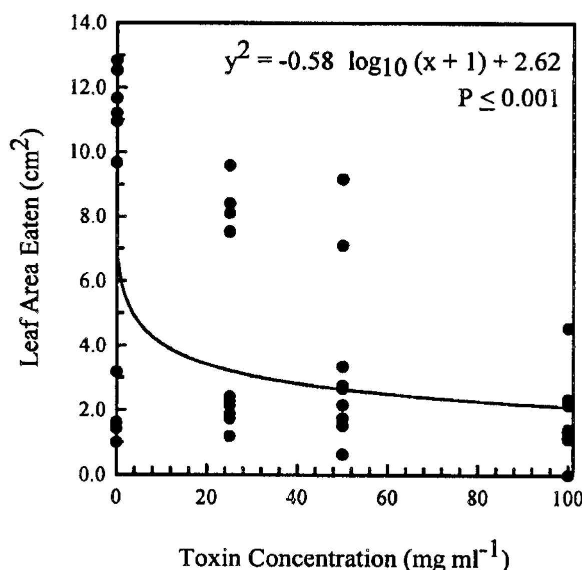

Fig. 5. Leaf area (cm') eaten by adult cottonwood leaf beetles when fed cottonwood leaf disks treated with different rates of Cry3A from a *Pseudomonas fluorescens* clone (B15). Each point represents the feeding of a

Table 4. Susceptibility of cottonwood leaf beetle populations to *B. thuringiensis* toxins cloned in P. fluorescens

Parameter measured	Toxin	Origin of insect population		
		Georgia	Virginia	Washington
% mortality[a] (SEM)	Cry3A	85 (0.1)a	86 (0.1)a	57 (0.1)b
	Cry3B	30 (0.1)a	19 (0.1)a	16 (0.1)a
Time to death[b] (d ± SEM)	Cry3A	2.8 (0.2)a	3.3 (0.3)a	5.3 (0.3)b
	Cry3B	8.1 (0.3)a	8.7 (0.3)ab	9.6 (0.3)b
Time to pupation of survivors[c] (d ± SEM)	Cry3A	10.4 (0.2)a	10.6 (0.2)a	10.5 (0.1)a
	Cry3B	10.4 (0.1)a	10.2 (0.1)a	9.9 (0.1)b
	Water control	9.7 (0.1)b	9.5 (0.1)c	9.1 (0.1)d

First instars were fed leaves dipped in 10 μg toxin ml^{-1} water. Treatment mortality was adjusted to account for control mortalities (Abbott 1925). Numbers in a row which are not followed by the same letter are significantly different ($P \leq 0.05$). For mean development time, numbers within a column that are not followed by the same letter are also significantly different ($P \leq 0.05$).

[a] Mean of 3 replicates with 50 insects each.

[b] Mean of those insects that died of 150 tested.

[c] Mean of those insects that survived of 150 tested.

egies involve providing some form of toxin-free refuge for susceptible pests (e.g., Raffa 1989, Tabashnik 1994, Wearing and Hokkanen 1995).

The low toxicity of *B.* thuringiensis on adults may prove beneficial for resistance management strategies in transgenic plantations. As with the Colorado potato beetle, the target pest for genetically engineered *B.* thuringiensis-potato varieties (Along et al. 1993, Perlak et al. 1993), adults present 2 problems. First, they cause significant damage to the crop (unlike Lepidoptera). Second, for refuge strategies to work, random mating must occur between adults from refuge and nonrefuge areas. If extensive intermating does not occur, small populations may develop resistance (Diehl and Bush 1984) . For those species in which the adult feeds, susceptible adults can move into the nonrefuge areas and mate randomly with resistant survivors only if they are not killed when on toxic plants.

In our experiments, cottonwood leaf beetle susceptibility varied among 3 widely separated populations, likely because of genetic variation, but possibly because of some environmental factor (such as an undetected 2nd pathogen). The Washington strain was the least susceptible, and it came from the fiber farm that had been managed for the longest period of time. The pesticides most commonly used to control cottonwood leafbeetles in this region are dimethoate and carbaryl. Cross-resistance between traditional pesticides and *B.* thuringiensis has not been identified in other insects (Everich et al. 1992), but the possibility cannot be ruled out here.

Van Frankenhuyzen et al. (1995) did not find populations of spruce budworm, *Choristoneura f*umiferana (Clemens) (Lepidoptera: Tortricidae), to differ significantly in their susceptibility to *B. thuringiensis,* although variation within populations was significant. We sampled only 3 populations, but *B, thuringiensis* susceptibility in the cottonwood leaf beetle showed more regional differences than has been found in the spruce budworm. If the differences we saw in susceptibility were genetic, regional differences in both the efficacy of transgenic poplars and the rate of B. thuringiensis-resistance development may occur in the field.

In conclusion, we are convinced that B. *thuringiensis* toxins are worth pursuing further for the development of genetically engineered insect resistance in Populus. However, once trees are successfully produced, greenhouse and field trials are needed to determine their efficacy. In addition, a combination of field trials and mathematical modeling of different management strategies are needed to develop sound resistance prevention systems if such trees are to be used in large production systems.

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